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Characterization of cells from pannus-like tissue over articular cartilage of advanced osteoarthritis

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Summary

Objective: To identify the characteristics of cells isolated from pannus-like soft tissue on osteoarthritic cartilage (OA pannus cells), and to evaluate the role of this tissue in osteoarthritis (OA).

Methods: OA pannus cells were isolated from pannus-like tissues in five joints obtained during arthroplasty. The phenotypic features of the isolated cells were characterized by safranin-O staining and immunohistochemical studies. Expression of MMP-1, MMP-3 and MMP-13 was also assessed using reverse transcriptase-polymerase chain reactions (RT-PCR), enzyme-linked immunosorbent assay (ELISA) and immunocytochemistry.

Results: Foci and plaque formation of pannus-like tissue over cartilage surface were found in 15 of 21 (71.4%) OA joints macroscopically, and among them, only five samples had enough tissue to be isolated. OA pannus cells were positive for type I collagen and vimentin, besides they also expressed type II collagen and aggrecan mRNA. Spontaneous expression of MMP-1, MMP-3 and MMP-13 was detected in OA pannus cells. Similar or higher levels of MMPs were detected in the supernatant of cultured OA pannus cells compared to OA chondrocytes, and among these MMP-3 levels were relatively higher in OA pannus cells. Immunohistochemically, MMP-3 positive cells located preferentially in pannus-like tissue on the border of original hyaline cartilage.

Conclusion: Our results showed that OA pannus cells shared the property of mesenchymal cells and chondrocytes; however, their origin seemed different from chondrocytes or synoviocytes. The spontaneous expression of MMPs suggests that they are involved in the articular degradation in OA.

Key words: Osteoarthritis, Pannus, Cartilage, MMP.

Introduction

Osteoarthritis (OA) is the most common form of arthropathy, and the major cause of disability in the elder. Histological studies of osteoarthritic joints show that the prominent change is the loss of hyaline cartilage from part or whole of the articular surface, accompanied by new bone formation (osteophyte). In addition, foci and plaques of new non-osseous tissue were often seen at the surface of diseased joints, which was considered as a phenomenon of remodeling process[1]. The histological appearance of the new tissue is described as a pannus-like fibrous tissue, chodroid, fibrocartilage or a form of hyaline cartilage[1–3]. Pannus is now known as invasive granulation tissue observed on articular surface of rheumatoid arthritis (RA) joints; however, the word 'pannus' originated in appearance like cloth that covered articular surface. Contrary to RA pannus, which had been extensively studied[4], little attention has been paid to the non-cartilaginous tissue covering OA cartilage. Recently, Shibakawa *et al.* reported that 80% of articular samples from OA patients had pannus-like tissue that had catabolic properties[5].

Matrix metalloproteinases (MMPs) play an important role in degeneration of articular cartilage in OA[6,7]. Recent studies have shown that MMP-1, MMP-3 and MMP-13 were present in synovial fluid (SF) from OA patients[8–10], and the severity of cartilage damage corresponded with synovial MMP activity[11]. By detecting degraded products of type II collagen and proteoglycan, the presence of specific MMP activity was elucidated *in vivo*[12–14]. In OA, the production of these MMPs was upregulated in synovial tissue[15,16], in SF cells[17], in chondrocytes[18–20] and in pannus-like tissue on OA cartilage[5].

In this study, we isolated cells from the pannus-like tissue on advanced OA cartilage (OA pannus cells) and cultivated them to investigate their phenotype and function. The production and expression of collagen, aggrecan and MMPs (MMP-1, MMP-3, and MMP-13) by these cells were evaluated and compared to that of chondrocytes from the same cartilage samples.

Patients and methods

CARTILAGE SAMPLES

Articular cartilage specimens were obtained from 21 patients who met the criteria of American College of Rheumatology for OA[21] and underwent arthroplasty.

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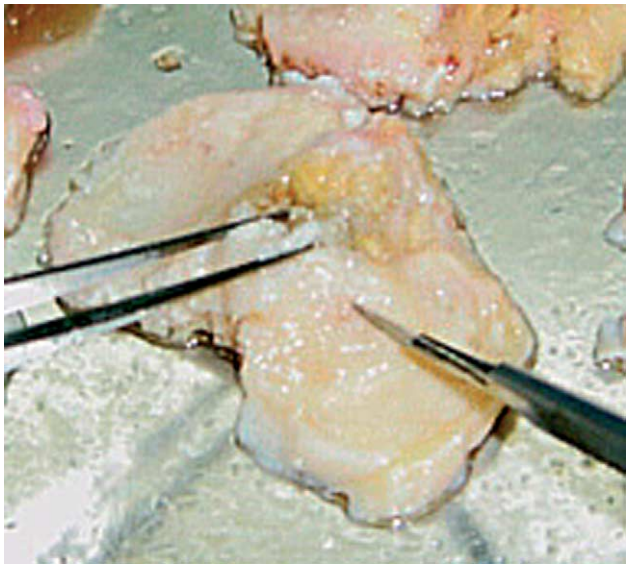

Fig. 1. Collection of pannus-like tissue from articular surface of lateral tibial condyle of the knee sample. The tissue was removed bluntly and gently from residual cartilage using a scalpel.

All the samples were from medial type knee OA or hip OA in advanced stage. Macroscopically, 15 of the 21 (71.4%) specimens had pannus-like tissue varying in size and locations, and we used 5 of the 15 specimens, which had enough tissue to be isolated (four knee joints and one hip joint, four females and one male, mean age of 76 years ranging from 56 to 94 years). All the samples were obtained with informed consent from the patients, and the study protocol was approved by the institutional ethical committee.

CELL PREPARATION

Pannus-like tissue was grazed off bluntly and gently from the residual hyaline cartilage as a thick material (Fig. 1). As pannus-like tissue occupied less than 30% of the surface of residual cartilage in OA with little quantity, it was difficult to detach enough samples discriminating two tissue types, fibrous type and vascular type, both of which were previously found[5]. Attention was paid not to scrape residual cartilage to cause contamination. OA pannus cells were prepared as follows. Pannus-like tissue was minced and then digested by rotating overnight at 37°C in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY) containing 1 mg/ml of bacterial collagenase (Sigma, MO). A part of the removed tissue was analyzed histologically to confirm that no piece of hyaline cartilage was included. The cells released by enzymatic digestion were filtered through a sterile nylon strainer (Becton Dickinson, NJ), washed, and centrifuged. The pellet was seeded into a 90 mm diameter dish (Sumitomo Medical Co., Tokyo) at a density of $2–4\times10^5$–4×10^5 cells/dish and cultivated at 37°C in a humidified atmosphere of 5% CO_2. The culture medium used was DMEM containing 10% heat-inactivated fetal calf serum (FCS; Gibco BRL) and 1% penicillin/streptomycin (Gibco BRL). Medium was replaced every week, and cells were used within the second passages. Chondrocytes were isolated from the residual hyaline cartilage taken from the articular region that was not covered with soft tissue. Fibroblast-like synoviocytes from synovium of the same samples were used as controls.

Table I
Primer sequences used for reverse transcription-polymerase chain reaction

Primer sequences (5′–3′)	Fragment size (bp)
Type II procollagen Sense: 5′-AACTGGCAAGCAAGGAGACA-3′ Antisense: 5′-AGTTTCAGGTCTCTGCAGGT-3′	621
Aggrecan Sense: 5′-ATGCCCAAGACTACCAGTGG-3′ Antisense: 5′-TCCTGGAAGCTCTTCTCAGT-3′	501/318
Type I procollagen Sense: 5′-TGACGAGACCAAGAACTG-3′ Antisense: 5′-CCATCCAAACCACTGAAACC-3′	599
MMP-1 Sense: 5′-CTGAAGGTGATGAAGCAGCC-3′ Antisense: 5′-AGTCCAAGAGAATGGCCGAG-3′	428
MMP-3 Sense: 5′-CCTCTGATGGCCCAGAATTGA-3′ Antisense: 5′-GAAATTGGCCACTCCCTGGGT-3′	440
MMP-13 Sense: 5′-CTATGGTCCAGGAGATGAAG-3′ Antisense: 5′-AGAGTCTTGCCTGTATCCTC-3′	390
GAPDH Sense: 5′-CCACCCATGGCAAATTCCATGGCA-3′ Antisense: 5′-TCTAGACGGCAGGTCAGGTCCA CC-3′	598

SAFRANIN-O STAINING AND IMMUNOSTAINING

Cells were seeded in eight-well chamber slides (Nunc, Naperville, IL) at a concentration of 2×10^4 cells per well and were cultured for 3–5 days. The slides were fixed using 4% formaldehyde in phosphate buffered saline (PBS) containing 0.1% saponin at room temperature for 30 min. Safranin-O staining was performed according to the method described previously[22]. Immunostaining was performed using HistoMark kits (streptavidin–horseradish peroxidase; KPL, Gaithersburg, MD). After fixation, slides were preincubated with heat-inactivated 10% goat serum at room temperature for 30 min, and then incubated at room temperature for 1 h with mouse anti-human collagen type I, collagen type II (Chemocon Int. Inc., Temecula, CA, 20 µg/ml), vimentin (Sigma, MO, 1:200) or anti-human MMP-3 (Fuji-Chemical Co., Japan, 10 µg/ml) monoclonal antibodies. Slides were washed twice with 0.04 M Tris buffered saline (pH 7.6) containing 0.1% saponin, then sequentially incubated twice with a biotinylated goat anti-mouse immunoglobulin and streptavidin–peroxidase conjugate at room temperature for 30 min. The streptavidin–peroxidase was then visualized using diaminobenzidine. Slides were counterstained with hematoxylin and evaluated with a bright-field microscope. For histochemical or immunohistochemical study, 5 µm thick sections of paraffin-embedded specimens were prepared and subjected to staining as mentioned above.

DETECTION OF MMP-1, MMP-3, AND MMP-13 OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The levels of MMP-1, MMP-3 and MMP-13 in supernatants were evaluated with commercially available ELISA kits according to the instructions of the manufacturer (Amersham Pharmacia Biotech, UK). These assays were

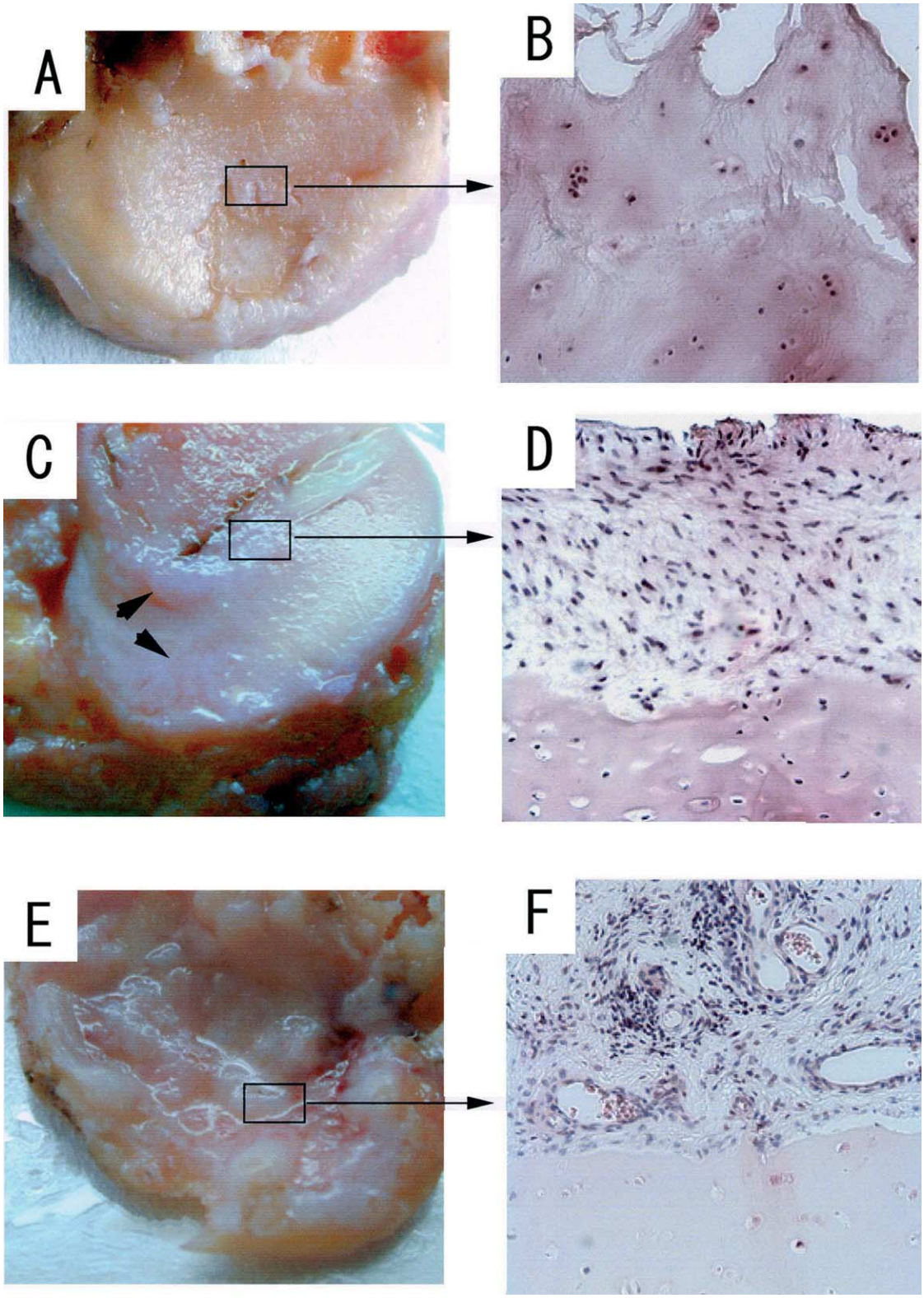

Fig. 2. Macroscopic (A, C and E) and microscopic (B, D and F; hematoxylin and eosin staining) views of articular surface of morbid joints. (A) and (B) show osteoarthritic knee joint without soft tissue from a 56-year-old male patient. Defect of cartilage is demarcated by distinct border of residual cartilage and lacerated surface is exposed directly. (C) and (D) show osteoarthritic knee joint with pannus-like tissue (arrowhead) from a 68-year-old female patient. Articular cartilage was partly covered with soft tissue like a cloth or a carpet. Histological finding is fibrous (fibrous type according to previous study[5]). (E) and (F) show rheumatoid arthritic knee joint from a 44-year-old patient with RA pannus. Articular surface was covered with invasive soft tissue with local bleeding. Histological findings show hypervascularity and infiltration of inflammatory cells.

capable of measuring each free proMMP as well as active MMP–TIMP-1 complex.

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR) ANALYSIS

Total RNA was extracted from 1×10^6 isolated cells using the single-step guanidinium thiocyanate–phenol–chloroform method (RNAzol B solution; Tel-Test, TX). RNA was precipitated in ethanol, recovered by centrifugation, resuspended in 10 µl of sterile water, and evaluated spectrophotometrically for quantity and purity. First-strand complementary DNA (cDNA) was synthesized in a 20-µl reaction mixture containing 5 µg of total RNA, 2.5 mM of each dNTP, 1 mM of random hexamer primers, 40 units of ribonuclease inhibitor (RNasin; Toyobo, Tokyo, Japan), and 200 units of Superscript II RT (Gibco BRL), by incubation at 42°C for 2 h. The resulting cDNA (2 µl) was subjected to PCR using *Taq* DNA polymerase (TaKaRa, Japan) and the specific primers for Col I, Col II, aggrecan, and MMPs. PCR for Col I, Col II and aggrecan was performed for 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and MMPs were amplified in a protocol of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 22 cycles to yield amplification in the linear range, in a PCR thermal cycler (TaKaRa)[23]. In each experiment, amplification of cDNA for the housekeeping gene GAPDH was used as an internal standard. The primer sequences are listed in Table I [24–26]. PCR products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide (10 mg/ml), and bands were visualized and photographed under ultraviolet illumination. The intensity of signal was quantified by an image analyzer, FLA 2000 (Fuji Film, Japan).

STATISTICAL ANALYSIS

For statistical analysis, Wilcoxon signed-ranks test was used to compare paired values and Mann–Whitney test for non-paired samples.

Results

MACROSCOPIC AND MICROSCOPIC FINDINGS OF ARTICULAR SURFACE IN OA AND RA

Macroscopically, the pannus-like tissue spread as a foci or plaque over residual hyaline cartilage in some OA samples [Fig. 2(C)]. Though there were samples lacking soft tissue covering [Fig. 2(A and B)], pannus-like tissue was found at the margins of articular surface or in more central areas [Fig. 2(C)], unlike RA pannus that spreads diffusely and invasively all over the articular surface with local bleeding [Fig. 2(E)]. Whereas RA pannus showed hypervascularity with infiltrating inflammatory cells accompanied by lymph follicles [Fig. 2(F)], pannus-like tissue of OA had less vascularity and fibrous property [Fig. 2(D)]. As is shown in the previous report[5], pannus-like tissue in OA was divided into two tissue types, fibrous type and vascular type, and was negative for proteoglycan presented by safranin-O staining. Contrary to cells infiltrating RA pannus, cells in OA pannus tissue were negative for CD68 (data not shown). The thickness of pannus over OA cartilage varied from only a thin layer to up to 5 mm.

EXPRESSION OF TYPE I COLLAGEN, TYPE II COLLAGEN, VIMENTIN AND PROTEOGLYCAN IN OA PANNUS CELLS

The previous study showed that pannus-like tissue of OA expressed type I collagen but not type II collagen or proteoglycan[5]. In the present study, the expression of chondrocyte specific molecules, type II collagen and aggrecan, as well as type I collagen and vimentin was investigated in OA pannus cells by RT-PCR and immunocytochemistry.

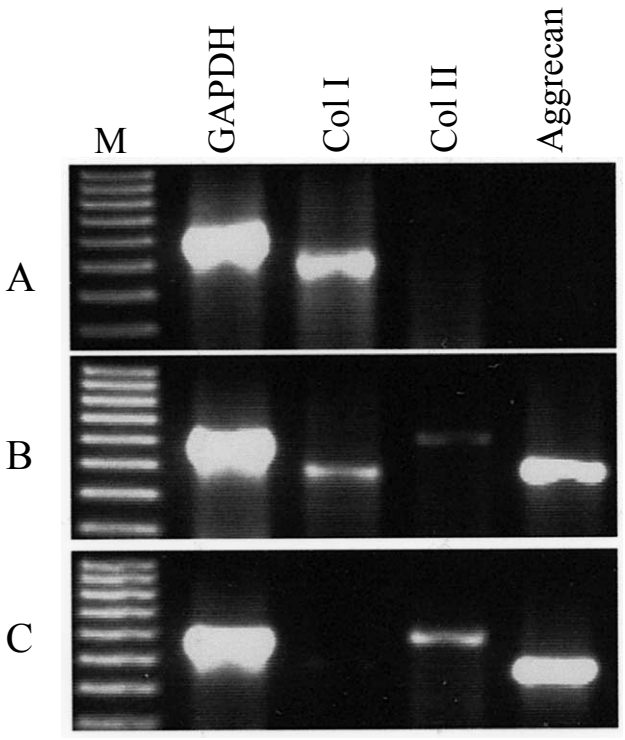

Fig. 3. RT-PCR image of type I collagen (Col I), type II collagen (Col II) and aggrecan expression in articular components of OA. Fibroblast-like synoviocytes (A), OA pannus cells (B) and chondrocytes (C). These cells were derived from the same patient.

RT-PCR showed that not only type II collagen and aggrecan, but also type I collagen was expressed in OA pannus cells in five samples obtained, though the intensity of type II collagen was relatively weak; on the other hand, chondrocytes expressed type II collagen and aggrecan, and synovial samples expressed only type I collagen in all samples tested. Figure 3 shows a representative result from five different samples.

In immunocytochemistry, OA pannus cells were faintly stained by safranin-O in two of the five samples; on the other hand, chondrocytes were strongly stained in all the samples. Moreover, OA pannus cells were positive for type I collagen, type II collagen and also vimentin (Fig. 4). Synoviocytes were only positive for type I collagen and chondrocytes were positive for safranin-O and type II collagen. Vimentin is a marker for cells of mesenchymal origin and positive for mesenchymal adherent cells in RA SF[27], whereas its expression was quite low in OA synoviocytes[28]. In this study, only OA pannus cells were positive for vimentin suggesting that they are of mesenchymal origin.

EXPRESSION AND PRODUCTION OF MMP-1, MMP-3 AND MMP-13 BY OA PANNUS CELLS

MMP-3 and IL-1β were positive in pannus-like tissue of OA, this tissue was speculated to have catabolic rather than anabolic property[5]. Thus we further investigated the

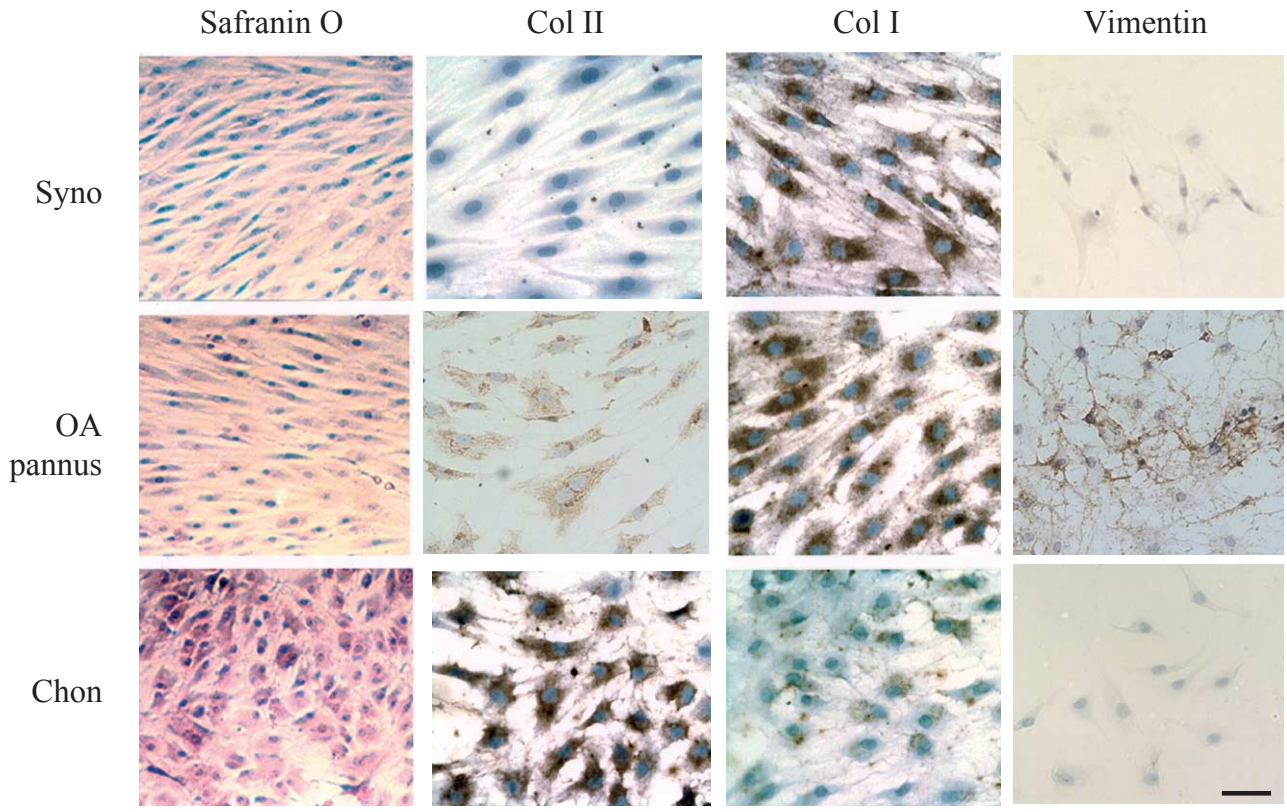

Fig. 4. Representative picture of safranin-O staining and immunostaining for type II collagen (Col II), type I collagen (Col I) and vimentin in articular components of OA. Fibroblast-like synoviocytes (Syno), OA pannus cells (OA pannus) and chondrocytes (Chon). Bar=50 μm.

expression and production of MMPs (MMP-1, -3 and -13) by isolated cells from the tissue.

RT-PCR analysis revealed that MMP-1, MMP-3 and MMP-13 were expressed in all OA pannus cell samples with various intensities [Fig. 5(A)]. In contrast, four of the five samples and only one sample expressed MMP-1 and MMP-3, respectively, in OA chondrocytes. All OA pannus cell samples expressed MMP-13. Although no significant difference was found in relative intensity, there was a tendency for MMP-3 to be higher and MMP-13 to be lower in OA pannus cells [Fig. 5(B)].

The expression of MMP-1, MMP-3 and MMP-13 in OA pannus cells was also confirmed by immunocytochemical study. OA pannus cells were revealed to express these MMPs and the expression of MMP-3 and -13 was stronger [Fig. 6(A–D)]. As MMP-3 was revealed to be the major MMP produced from isolated chondrocytes stimulated with IL-1 or TNF[29], *in vivo* localization was confirmed in three samples by immunohistochemistry. A representative picture showed that a considerable number of cells in pannus-like tissue were positive for MMP-3, while fewer chondrocytes were positive in hyaline cartilage underlying the tissue [Fig. 6(E)].

The production of MMP-1, MMP-3 and MMP-13 from OA pannus cells and chondrocytes in culture supernatant was also analyzed by ELISA. As shown in Fig. 7, the production of MMP-3 was significantly higher in OA pannus cells; however, no significant difference was shown in other MMPs. The levels of MMP-1 and MMP-13 were relatively low compared with those of MMP-3.

Discussion

Shibakawa *et al*. showed that pannus-like tissue was found on OA cartilage, preferentially located in the marginal region of the joint and could be classified into the vascular type and the fibrous type. From the detailed histological observation, they speculated that the tissue was reparative in the earlier stage of OA, but changed to be catabolic after the long-term exposure to factors such as cytokines or growth factors involved in OA[5]. Pannus-like tissue in OA was different from RA pannus in the following ways: (1) it often occurred as foci or plaque over cartilage surface, unlike RA pannus which distributed diffusely over the cartilage; (2) its cell density was less than that of RA pannus, especially on the border of original hyaline cartilage; and (3) in contrast to RA pannus, neither lymphocyte follicles nor CD68+ cells were found.

In regard to the incidence of pannus-like tissue, Meachim and Osborne examined 20 osteoarthritic femoral heads and reported that soft tissue, varying in pattern from fibrous to loose-textured, was found in all the specimens and occupied 10–55% of the diseased joint surface[1,2]. In our study, articular surface was observed macroscopically and pannus-like tissue was found in 71.4% of joint samples. The incidence may vary according to the stage of the disease and the joint examined. Anyway, the exact incidence of pannus occurring in early stage OA needs to be further investigated in arthroscopic study or using an animal model.

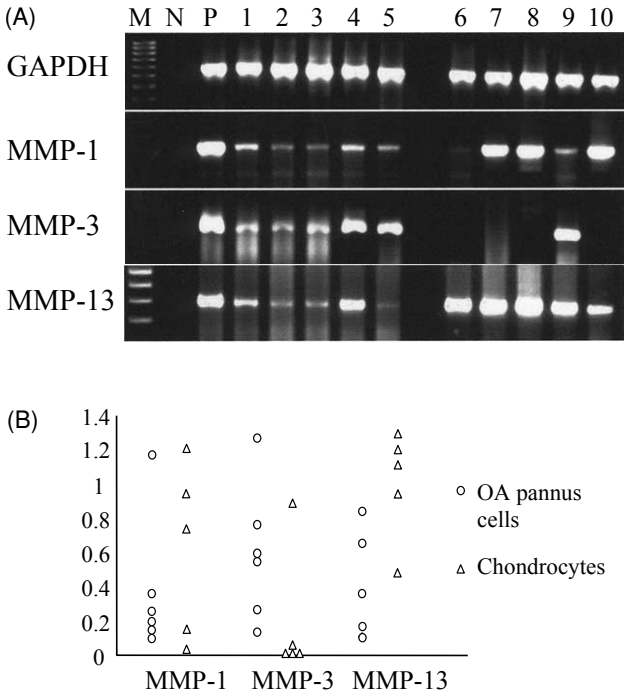

Fig. 5. (A) RT-PCR analysis for MMP-1, MMP-3 and MMP-13 expression in OA pannus cells (lanes 1–5) and chondrocytes (lanes 6–10) from OA patients. M, molecular weight marker; N, negative control (no template); P, positive control (TNFα-stimulated chondrocytes). (B) Intensity of the signals analyzed by an image analyzer. Values were intensity relative to that of GAPDH. Though no significant difference was found, there was a tendency for MMP-3 to be higher and MMP-13 to be lower in OA pannus cells.

As the property of pannus-like tissue was different from that of hyaline cartilage, we further investigated the cells isolated from this tissue. We found that OA pannus cells strongly expressed a fibroblastic marker, type I collagen; however, they also expressed type II collagen and aggrecan mRNA to some extent. Thus, OA pannus cells shared some properties of chondrocytes. OA pannus cells might include heterogeneous cells, unlike chondrocytes, considering the fact that pannus-like tissue of OA was classified into fibrous type and vascular type; however, *in situ* properties of both were similar[5].

As for the origin of pannus-like tissue of OA, it can spread over the joint surface from synovial tissue at the articular margins or it may be caused by fibrous metaplasia of cartilage as it shared the common property of chondrocytes. Another candidate is soft tissue of subarticular origin that gains access to the surface through gaps in the bone[1,2]. The fact that only OA pannus cells expressed vimentin, a mesenchymal marker, supports the possibility that its origin is subarticular mesenchymal cells.

As mentioned above, pannus-like tissue of OA had a catabolic property, although it is speculated to be reparative in the earlier stage of this disease. A previous report also described cartilage erosion by this tissue[30]. In this regard, it might have, partly, some common properties as RA pannus in cartilage destruction. In this study, we confirmed the expression of MMP-1 -3 and -13, and showed that OA pannus cells as well as OA chondrocytes expressed MMPs; especially, OA pannus cells produced more MMP-3 than OA chondrocytes at protein level. Distinctive expression of MMP-3 was found only in one sample by RT-PCR, which was inconsistent with the result from ELISA, as MMP-3 was detected in the culture supernatant of all samples as shown in Fig. 7. One of the reasons for this inconsistency might be insufficient amplification (22 cycles) to get the semi-quantitative results. MMP-3 expression was detected in OA chondrocytes amplified for 35 cycles (data not shown).

Since it is well known that these MMPs play a crucial role in cartilage damage by degrading cartilage components including collagen types II, IX, XI and proteoglycan[31,32], our results support the previous speculation that pannus-like

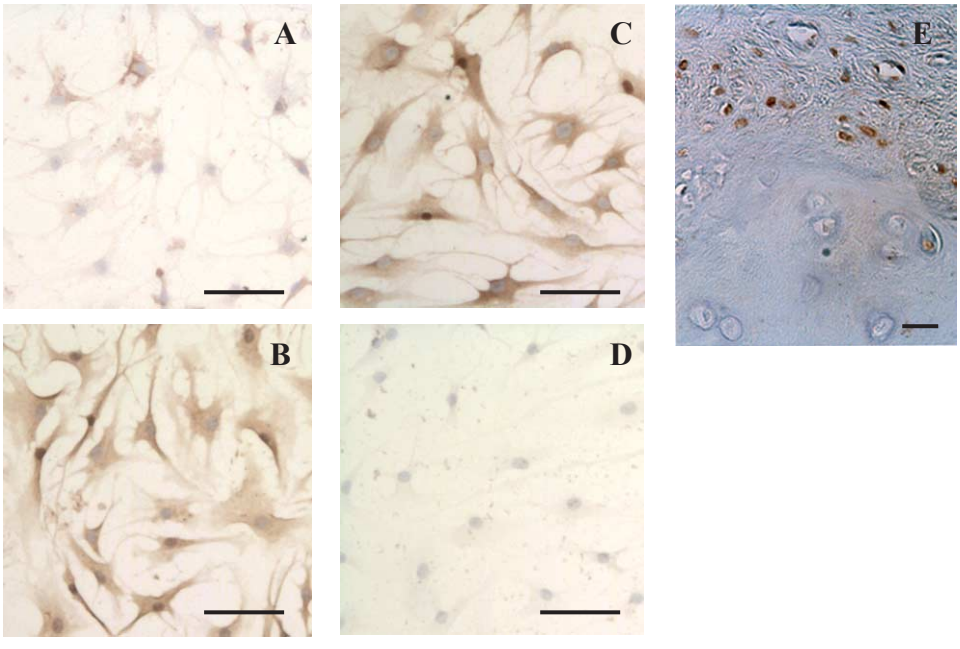

Fig. 6. Immunostaining of MMPs in OA pannus cells (A–D) and MMP-3 in OA cartilage (E). (A) MMP-1, (B) MMP-3, (C) MMP-13; (D) control.

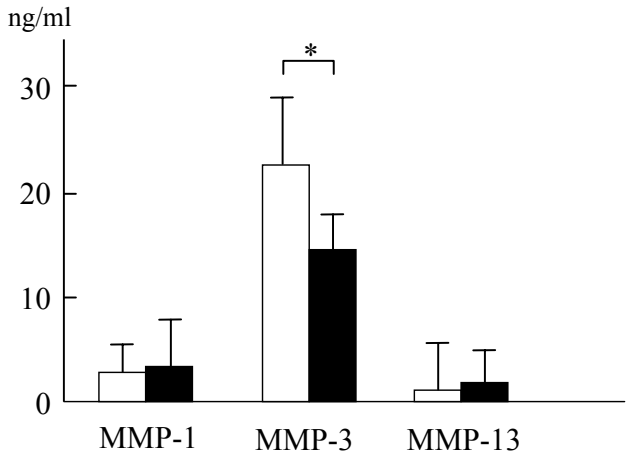

Fig. 7. MMP-1, MMP-3 and MMP-13 productions *in vitro* by OA pannus cells (white bar) and chondrocytes isolated from OA patients (black bar). Values are the concentration of each MMP in the supernatant of cultured cells and expressed as the mean and SD. Statistical analysis was performed by Wilcoxon signed-ranks test (*P<0.05).

tissue of OA is involved in further cartilage degradation, especially in advanced OA. This leads to the hypothesis that cartilage defects may be bridged by fibrous tissue for repair, but continual joint movement or unfavorable circumstances such as exposure to cytokines may make it unlikely for a repair process to succeed. The expression of proteoglycan and type II collagen suggests the possibility of repair by this tissue in some condition, even though catabolic factors are also expressed.

In conclusion, we demonstrated pannus-like tissue over OA cartilage, and the cells isolated from this tissue shared properties of mesenchymal cells and chondrocytes to some extent. In addition, OA pannus may be involved in cartilage degradation in OA by producing MMPs. Our results shed light on the role of soft tissue in cartilage degradation in OA, suggesting the possibility of new therapeutic strategy in OA, by targeting pannus-like tissue of OA to prevent cartilage from further degradation.

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